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Ly49A Transgenic Mice Provide Evidence for a Major Histocompatibility Complex–dependent Education Process in Natural Killer Cell Development

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Summary

The Ly49 natural killer (NK) cell receptors are class I MHC–specific inhibitory receptors that are distributed to overlapping NK cell subsets. The formation of the Ly49 receptor repertoire was examined with transgenic mice that express Ly49A in all NK cells. In MHC class I–deficient mice, the Ly49A transgene did not prevent expression of endogenous *Ly49* genes. However, in H-2^d mice that express a Ly49A ligand, the transgene caused clear alterations in the endogenous Ly49 repertoire. The frequency of NK cells expressing another H-2^d–specific receptor, Ly49G2^+, was substantially reduced. Reduced numbers of cells expressing endogenous *Ly49A* was suggested by reduced endogenous Ly49A mRNA levels. These results support the existence of an MHC-dependent education process that limits the number of NK cells that coexpress multiple self-specific Ly49 receptors. Ligand-dependent downregulation of Ly49 cell surface levels was also examined. Cell-surface downregulation occurred even when the transgene was expressed at low levels. The results demonstrate that downregulation of Ly49A cell surface levels is a posttranscriptional event, and argue against a model in which Ly49 receptors are calibrated to specific cell surface levels depending on the available class I ligands.

NK cells recognize a variety of target cells, including tumor cells, cells infected with some viruses or bacteria, and some normal cells. A critical determinant of target cell recognition by NK cells is the MHC class I expression pattern of the target cell. NK cells are equipped with receptors that display specificity for allelic variants of MHC class I molecules. In the human, these receptors have been identified as a family of proteins with homology to immunoglobulins (1–4). In the mouse, the class I–specific receptors are encoded by at least nine closely related genes which encode C type lectin-like Ly49 receptors (Ly49A-I) (5). The expression of Ly49 receptors is restricted to NK cells and a small subset of T cells (5–8). Monoclonal antibodies have been generated against the Ly49A, Ly49G2, and Ly49C/I receptors. Each of these antibodies defines a subpopulation of 20–50% of total NK cells. Coexpression of two or more receptors is quite common, as many NK cells can be costained with two or even all three antibodies (7, 9–12). The common coexpression of two or more receptors results in a complex Ly49 repertoire, despite the relatively small number of receptors.

The specificities of some Ly49 receptors have been investigated. The Ly49A receptor is specific for D^d and D^k class I molecules (13–15). The Ly49G2^+ subset is inhibited by target cells expressing D^d or L^d (10). The SW-5E6 mAb was originally thought to specifically define the Ly49C receptor (7, 11). However, recent evidence indicates that in B6 mice, SW-5E6 binds to both Ly49C and Ly49I (16). Based on cell binding studies, Ly49C binds to both H-2^b and H-2^d class I molecules, and Ly49I may bind to neither (16).

The distribution of Ly49 receptors to distinct, albeit overlapping, NK cell subsets has important biological consequences. In normal H-2^b mice, where ~20% of NK cells express Ly49A, lysis of H-2^d target cells is accomplished by Ly49A$^-$ NK cells (17). Expression of Ly49A in all NK cells from a transgene prevented H-2^b mice from rejecting H-2^d bone marrow grafts in vivo, and from lysing H-2^d tumor target cells in vitro (18), presumably because each NK cell was inhibited when it encountered D^d-expressing cells. Thus, the restriction of inhibitory Ly49 receptor expression to subsets of NK cells is a necessary condition to observe allo-aggression in the NK cell compartment. By the same reasoning, subset-specific expression of Ly49 receptors is likely to be necessary for NK cells to attack self cells that have extinguished expression of some, but not all, class I molecules as a consequence of infection or mutation.

Little is known concerning the mechanisms that impose subset-specific expression of Ly49 receptors. We recently reported that most NK cells in *Ly49A* heterozygous mice that express the *Ly49A* gene express only one or the other *Ly49A* allele (9). Monoallelic expression of *Ly49* genes can be accounted for by a number of different models. *Ly49* gene expression may be regulated by a feedback mechanism wherein the expression of Ly49A from one allele pre-

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vents expression of the other *Ly49A* allele. Such a mechanism could have evolved to prevent the coexpression of two allelic Ly49 receptors with distinct specificities. Indeed, *Ly49* genes exhibit allelic sequence polymorphism (9–11, 16). However, it is difficult to understand why coexpression of both alleles at the same locus would be prevented in NK cells, when coexpression of different *Ly49* genes in the same cell is quite frequent. An alternative explanation of monoallelic expression of *Ly49* genes is that it reflects the outcome of a more general process that distributes the expression of Ly49 receptors to different NK cells. It was proposed that a process governing receptor expression imparts a specific probability of stable activation to each *Ly49* allele in individual NK cells (9, 19). Most Ly49A$^+$ NK cells would thus express only one or the other *Ly49A* allele; a smaller number would simultaneously express both alleles.

Whatever the mechanisms that generate clonal diversity of Ly49 receptor expression in NK cells, it is likely that they are coordinated with "education" processes that adapt NK cells to the MHC environment in which they develop. Indeed, the results of functional experiments suggest that the NK cell population is adapted to the MHC molecules of the host (20–24). It was postulated that the education process ensures that each NK cell expresses at least one inhibitory receptor specific for at least one self–MHC class I allele, to avoid autoimmunity. Consistent with this hypothesis, cultured Ly49A$^+$ NK cells isolated from H-2^b mice were tolerant of H-2^b lymphoblast target cells, whereas Ly49A$^+$ NK cells from H-2^d mice efficiently lysed H-2^b target cells (25, 26). Both populations of NK cells lysed class I–deficient lymphoblasts equivalently, indicating that the Ly49A$^+$ cells from H-2^b mice were not simply anergic. Furthermore, antibody fragments specific for Ly49A did not restore lysis of H-2^b target cells by Ly49A$^+$ NK cells from H-2^b mice, suggesting that inhibition was not mediated through Ly49A, and therefore that these NK cells expressed additional, H-2^b–specific receptors compared to the Ly49A$^+$ NK cells from H-2^d mice (26). Consequently, although definitive evidence has not yet been obtained, it seems that each functional NK cell expresses at least one self class I–specific Ly49 receptor.

In addition to the functional evidence discussed above, we have reported that expression of MHC class I molecules affects the frequencies of Ly49-defined subsets. NK cells that matured in an environment of low MHC class I expression in $\beta 2m^{-/-}$ mice included a higher frequency of cells expressing two or three different Ly49 receptors than did NK cells from normal $\beta 2m^+$ mice (12). Moreover, it was observed that NK cells expressing multiple H-2^d–specific Ly49 receptors were significantly less frequent in H-2^d mice that express the corresponding inhibitory MHC ligand, than in H-2^b mice (12). These observations suggested that an education process dependent on class I MHC limits the number of different Ly49 receptors expressed by individual NK cells.

MHC class I molecules affect not only the frequencies of cells expressing specific Ly49 receptors, but also the cell surface levels of Ly49 receptors. Studies performed with Ly49A (13, 25) and Ly49G2 (12) indicated that these receptors are expressed at lower cell surface levels in the presence of their inhibitory MHC ligand compared to its absence. This finding led to the suggestion that a developmental process calibrates the expression level of the inhibitory receptor to the respective MHC environment, allowing NK cells to detect small alterations in the levels of self–class I molecules (25).

The hypothesis that an education process limits the numbers of cells that express multiple self-specific Ly49 receptors leads to the prediction that expression of a Ly49A transgene in all NK cells of H-2^d mice will result in a reduced frequency of cells expressing endogenous receptors of the same specificity. Here we have tested this hypothesis. Expression of an Ly49A transgene causes a reduction in the usage of a second H-2^d–specific Ly49 receptor in H-2^d mice, but not in class I–deficient mice, strongly supporting the existence of an education process that limits Ly49 receptor coexpression. Furthermore, we used the transgenic mice to test the hypothesis that monoallelic expression of *Ly49A* genes is the result of a mechanism wherein the mere presence of Ly49A protein expressed from one allele prevents expression of the other Ly49A allele. It was observed that the ubiquitously expressed Ly49A transgene did not prevent expression of the endogenous *Ly49A* gene in class I–deficient mice, arguing against this model. The transgenic mice also provide a means to investigate the mechanisms that underlie the reduction of Ly49A levels observed in mice that express Ly49 ligands. The results indicate that the ligand-induced reduction in Ly49A levels is largely determined by a posttranscriptional process, and that down-regulation is observed, even when the levels of transgene-encoded Ly49A are low to begin with. These results argue against a model in which Ly49A levels are adjusted to a specific level depending on ligand binding.

Materials and Methods

Mice. B10.D2/oSnJ were purchased from the Jackson Labs (Bar Harbor, ME) or the National Cancer Institute (Frederick, MD). C57BL/6J (B6) mice were bred in the animal facility of the University of California (Berkeley, CA). Class I MHC–deficient (B6 $\beta 2m^-$) mice were established by backcrossing (129/Sv $\times$ B6)F1 $\beta 2m^-$ mice to B6 mice five times and intercrossing.

Generation and Analysis of Ly49A Transgenic Mice. The generation of Ly49A transgenic mice is described in detail elsewhere (18). In brief, a Ly49A cDNA clone was isolated from the C57/BL6 (B6)–derived thymoma EL-4 using reverse transcriptase PCR with Ly49A-specific primers (9). The Ly49A cDNA clone was subcloned into the class I promoter expression cassette (27) and injected into fertilized (B6 $\times$ CBA/J)F_2 eggs to generate transgenic mice.

Transgenic founder mice ([B6 $\times$ CBA/J]F2 mice) were first backcrossed three times to B6 mice, and offspring were typed for the segregation of H-2 alleles with antibodies specific for H-2D^b (28-14-8s; reference 28) or H-2K^k (PharMingen, San Diego, CA), as well as the NK complex with the B6 allele-specific mAb PK136 (anti-NK1.1) (29). Subsequently, transgenic mice were crossed to B10.D2 mice to generate H-2$^{b/d}$ and H-2^d transgenics, and to B6 $\beta 2m^{-/-}$ mice to generate $\beta 2$m-deficient Ly49A transgenics. In

the experiments shown here, all mice used were hemizygous for the Ly49A transgene, had been backcrossed three times to the B6/B10 background, the MHC haplotypes were H-$2^{b/b}$, H-$2^{b/d}$, H-$2^{d/d}$, or $\beta2m^{-/-}$, as indicated, and the NK complex on chromosome 6 was always homozygous of B6 origin.

Flow Cytometry. Spleen cell suspensions from individual mice were passed over nylon wool columns and nonadherent cells (5×10^5) were stained with PE-labeled goat anti–mouse IgG (Southern Biotechnology Assoc., Birmingham, AL). Free binding sites were saturated with mouse IgG. Cells were further stained with PE-conjugated anti-CD3 (PharMingen, San Diego, CA) and biotinylated PK136 (anti-NK1.1). After washing, the cells were incubated with a mixture of streptavidin-Tricolor (CALTAG, San Francisco, CA) plus FITC-labeled JR9-318 (JR9; anti–Ly49A), SW5E6 (anti-Ly49C), or 4D11 (anti-Ly49G2). In general, 5×10^4–10^5 events were analyzed on a flow cytometer (EPICS XL; Coulter Corp., Hialeah, FL). Expression of Ly49 receptors on NK cells was assessed by gating on NK1.1$^+$CD3$^-$ Ig$^-$ cells. Graphics were generated using the WindMDi software (John Trotter, Salk Institute, La Jolla, CA).

Ribonuclease Protection Assay. The 3′ portion of Ly49A cDNA (position 783-1162, reference 30), most of which is not included in the Ly49A transgene construct (903-1162), and an NK1.1 (NKR-P1C) fragment (207-620, clone No. 40, reference 31), were obtained by PCR from B6 A-LAK cDNA using the following Ly49A- and NKRP1-specific PCR primers, respectively: Ly49A, 804 sense: GGAAATACAATATAAGAGATGGG; 1143 antisense: CAAATAAAACAGATTCGTCC; NKR-P1 (NKR-P1A and C), 207 sense: ACACAGGTTGGCTCTGAAGC, and (NKR-P1B and C), 680 antisense: ACTTTRTCTCCTRAGATGGWG.

PCR products were cloned into T vectors and sequenced to confirm identity with the published sequences. To synthesize the ^{32}P-labeled probes for RNase protection, the Ly49A construct was linearized with EcoRI and the NK1.1 construct was cut with BstX II resulting in a shortened NK1.1 probe (381-620) after transcription with T7 RNA polymerase.

Day 3 A-LAK cells from $\beta2m^-$, H-2^b, or H-2^d Ly49A transgenic mice and their nontransgenic littermates were used as a source of total cellular RNA for the RNase protection assay. For each sample, the RNA equivalent of 10^6 NK1.1$^+$ cells (usually 70–80% of total cells) was analyzed simultaneously for Ly49A and NK1.1 mRNA as described (32). The protected fragments after RNase digestion (379 nucleotides (nt)[1] for the endogenous Ly49A, 120 nt for the Ly49A transgene, and 239 nt for NK1.1) were separated on a 4% denaturing polyacrylamide gel. Signal quantitation was performed using a PhosphorImager SF (Molecular Dynamics, Inc., Sunnyvale, CA).

Results

Ly49A Cell Surface Levels in Transgenic Mice. We have generated transgenic mice expressing a Ly49A cDNA of B6 origin, driven by the class I promoter and the immunoglobulin enhancer. A previous analysis of an H-2^b transgenic line (line No. 2) with a high (>15) transgene copy number demonstrated that the transgenic Ly49A receptor was expressed on the surface of essentially all NK cells, T cells, and B cells, and the level of expression was similar to that of normal NK cells (Fig. 1, Table 1; reference 18). Transgene expression conferred D^d-specific inhibitory function to NK cells, as tested in vitro or in vivo (18). In a second lower copy transgenic line (line No. 12), the level of Ly49A expression on the surface of most of the NK cells from H-2^b mice was ~30% of the normal level (Fig. 1, Table 1), whereas some NK cells, presumably those expressing endogenous Ly49, continued to express high levels of Ly49A (Fig. 1).

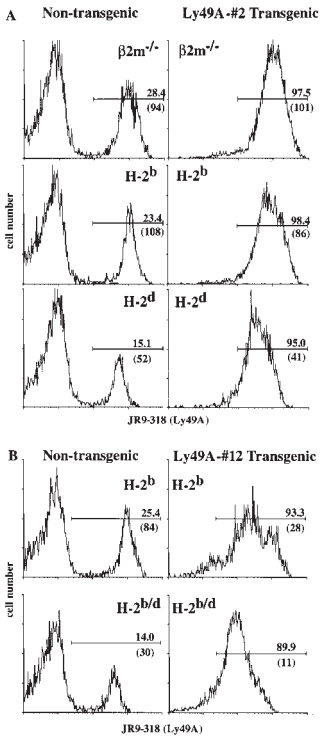

Figure 1. Analysis of Ly49A expression in MHC different, Ly49A transgenic mice. Gated NK1.1$^+$ CD3$^-$ splenocytes from transgenic line No. 2 (*A*), or transgenic line No. 12 (*B*) of the indicated MHC types were compared with nontransgenic littermates after staining with Ly49A-specific mAb JR9-318. In nontransgenic mice, Ly49A is expressed on an NK cell subset, the size of which is usually smallest in H-2^d mice (see also reference 12), whereas all transgenic NK cells express Ly49A. The frequency of stained cells and the mean fluorescence intensity (*in parentheses*) are indicated.

Previous studies in normal mice have demonstrated that the cell surface levels of Ly49A, on NK cells are lower in mice that express the D^d ligand for Ly49A, than in H-2^b or $\beta2m^{-/-}$ mice (12, 13, 25). Here we determined the effects of MHC genes on the cell surface levels of the transgene-encoded Ly49A receptor. We transferred the transgenes onto different MHC backgrounds by backcrossing to B6, B10.D2, or B6-$\beta2m^{-/-}$ strains. Irrespective of their MHC background, transgenic mice and their nontransgenic littermates were found to contain comparable numbers of NK cells per spleen (data not shown), indicating that expression of the Ly49A transgene did not prevent NK cell development, even in the presence of the inhibitory H-2^d ligand of Ly49A. In Ly49A transgenic line No. 2 mice, Ly49A cell surface levels on all NK cells were reduced by a factor of two- to threefold in H-2^d mice, compared to the levels in H-2^b or $\beta2m^{-/-}$ mice (Fig. 1, Table 1). Although Ly49A transgenic line No. 12 mice had generally lower levels of Ly49A cell surface expression than did line No. 2 mice, H-2^d expression (in H-$2^{b/d}$ mice) nevertheless also caused a two- to threefold reduction in Ly49A levels compared to levels in H-2^b mice (Fig. 1, Table 1).

The magnitudes of these effects were similar to the magnitude of the reduction observed for the endogenous Ly49A

[1] *Abbreviation used in this paper:* nt, nucleotides.

Table 1. *Expression Levels of Ly49 Receptors in Ly49A Transgenic Mice*

		Mean Fluorescence Intensity		
	MHC	mAb JR9-318 (Ly49A)	mAb SW5E6 (Ly49C/I)	mAb 4D11 (Ly49G2)
Tg line No. 2	$\beta 2m^{-/-}$	97 ± 9	55 ± 4	19 ± 2
	$H\text{-}2^b$	94 ± 12	49 ± 2	19 ± 1
	$H\text{-}2^d$	**35 ± 4**‡	36 ± 2‡	10 ± 1‡
non-Tg LM	$\beta 2m^{-/-}$	109 ± 22	56 ± 1§	19 ± 4
	$H\text{-}2^b$	105 ± 7	48 ± 3	18 ± 1
	$H\text{-}2^d$	48 ± 4*	39 ± 3§	12 ± 1‡
Tg line No. 12	$H\text{-}2^b$	30 ± 4	40 ± 1	16 ± 1
	$H\text{-}2^{b/d}$	**13 ± 1**‡	33 ± 2*	11 ± 1*
non-Tg LM	$H\text{-}2^b$	90 ± 3	46 ± 3	16 ± 2
	$H\text{-}2^{b/d}$	36 ± 2*	37 ± 3‡	11 ± 1‡

Values represent the means and SDs of three determinations. The nontransgenic samples were from littermates of the respective transgenic lines, analyzed in parallel. The two transgenic lines were tested in separate experiments. In the case of Ly49A transgenic mice, Ly49A staining intensity was determined by gating on all NK cells. In the case of nontransgenic littermates, Ly49A staining intensity was determined by gating on $Ly49A^+$ NK cells. Substantial effects of MHC on transgene-directed Ly49A levels are indicated in bold. *LM*, littermate; *Tg*, transgenic.
*$P < 0.001$; ‡$P < 0.01$; §$P < 0.05$, compared to $H\text{-}2^b$ mice of same type by Student's *t* test.

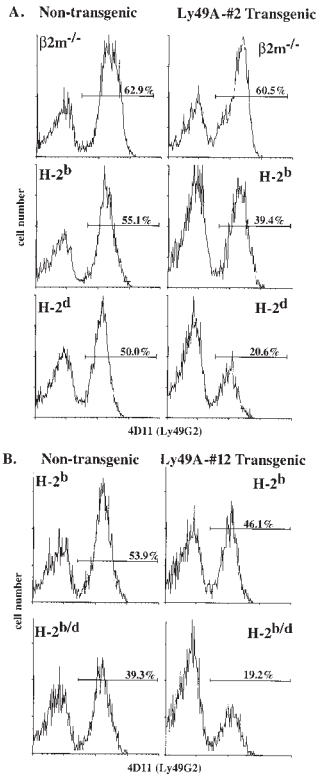

Figure 2. The Ly49A transgene reduces the frequency of $Ly49G2^+$ cells in an MHC class I–dependent fashion. Gated $NK1.1^+$ $CD3^-$ nylon wool nonadherent splenocytes from transgenic line No. 2 (*A*), or transgenic line No. 12 (*B*) of the indicated MHC types were compared with nontransgenic littermates after staining with Ly49G2-specific mAb 4D11. The frequency of stained cells is indicated.

in nontransgenic mice (Fig. 1, Table 1). The results indicate that Ly49A expression driven by the class I promoter cassette is subject to ligand-mediated downregulation, and therefore, that receptor downregulation does not require the *Ly49A* gene regulatory elements. The fact that the low cell surface level of the transgene-encoded Ly49A in $H\text{-}2^b$ line No. 12 mice is reduced even further by $H\text{-}2^d$ expression indicates that the levels are reduced from the level in the absence of ligand, rather than being adjusted to a specific level determined by the MHC ligand.

The Ly49A Transgene Does Not Inhibit Expression of Endogenous Ly49 Receptors in $\beta 2m^{-/-}$ Mice. The expression of a TCR-β transgene in mice prevents the expression of endogenous TCR-β genes, even in mice that do not provide a selecting MHC ligand for the transgenic receptor. To ascertain whether Ly49A transgene expression inhibits expression of endogenous *Ly49* genes, we determined whether the transgene influenced the frequencies of NK cells expressing receptors reacting with the SW5E6 mAb (Ly49C/I) and 4D11 (Ly49G2) mAbs. In β2m-deficient mice, the Ly49A transgene (line No. 2) had no detectable effect on the frequencies of $SW5E6^+$ or $4D11^+$ NK cells (Fig. 2, Table 2), nor did the transgene cause alterations in the staining intensities observed with these mAbs (Table 1). Thus, the Ly49A transgene did not appreciably inhibit endogenous Ly49G2 or Ly49C/I expression in class I–deficient mice.

The frequency of cells expressing the endogenous *Ly49A* gene could not be determined by flow cytometry because all Ly49A antibodies react with the B6 Ly49A protein used as the transgene. However, the transgenic and endogenous Ly49A mRNAs contained distinct 3′ untranslated regions, allowing us to determine the levels of endogenous Ly49A mRNA with the ribonuclease protection assay (33). When an internal control probe is used, this assay is one of the most quantitative assays for determining mRNA levels (33). The use of a large excess of probe makes the assay linear with respect to mRNA levels. A probe was designed that spanned the 3′ end of the coding region and part of the 3′ untranslated region of the Ly49A mRNA, a region not included in the transgene construct. A probe for the NKR-P1C mRNA served as an internal control to which the Ly49A signal was normalized. NKR-P1C encodes the NK1.1 antigen, which is expressed by all NK cells in the mouse strains used here (34). Representative data are shown in Fig. 3, and the results from three experiments are summarized in Table 3.

In β2m-deficient and $H\text{-}2^b$ mice, the Ly49A transgene did not appreciably inhibit expression of the endogenous Ly49A mRNA (Fig. 3, Table 3). These findings indicate that the mere presence of the transgene-encoded Ly49A protein in the cell does not prevent expression of the endogenous *Ly49A* gene. Monoallelic *Ly49A* gene expression in normal mice must therefore be explained by a distinct mechanism. As will be elaborated below, the transgene did influence endogenous *Ly49A* gene expression in $H\text{-}2^d$ mice.

Ly49A Transgene Biases the Endogenous Repertoire in $H\text{-}2^d$ Mice. The Ly49A transgenic mice allowed us to test the hypothesis that education processes limit the frequencies of

Table 2. *The Size of Ly49-defined NK Cell Subsets Is Influenced by Ly49A Transgene and MHC*

		Percentage of NK1.1$^+$CD3$^-$ cells		
	MHC	mAb JR9-318 (Ly49A)	mAb SW5E6 (Ly49C/I)	mAb 4D11 (Ly49G)
Tg line				
No. 2	β2m$^{-/-}$	96.7 ± 2.3	64.8 ± 1.9	57.2 ± 6.6
	H-2^b	98.0 ± 0.9	45.6 ± 5.0	38.5 ± 3.0*
	H-2^d	93.8 ± 1.3	47.2 ± 2.8‡	**18.5 ± 1.8***
non-Tg LM	β2m$^{-/-}$	31.8 ± 3.6	64.2 ± 1.3	63.5 ± 0.1
	H-2^b	21.3 ± 3.4	48.4 ± 5.0	51.0 ± 3.9
	H-2^d	15.5 ± 3.1	55.1 ± 0.7	50.7 ± 3.3
Tg line				
No. 12	H-2^b	90.2 ± 1.7	46.5 ± 4.5§	46.1 ± 3.1‡
	H-2$^{b/d}$	89.6 ± 2.7	42.6 ± 3.2‡	**22.3 ± 3.1***
non-Tg LM	H-2^b	24.5 ± 1.6	52.9 ± 1.9	54.7 ± 0.5
	H-2$^{b/d}$	15.1 ± 3.4	49.2 ± 2.1	45.7 ± 4.4

Data represent means of three or more determinations ± SDs. The nontransgenic samples were littermates of the respective transgenics and were tested in parallel. Substantial effects of transgene expression on Ly49G2 usage are indicated in bold.
*P <0.001; ‡P <0.01; §P <0.05, compared to nontransgenic mice of same MHC type by Student's t test.

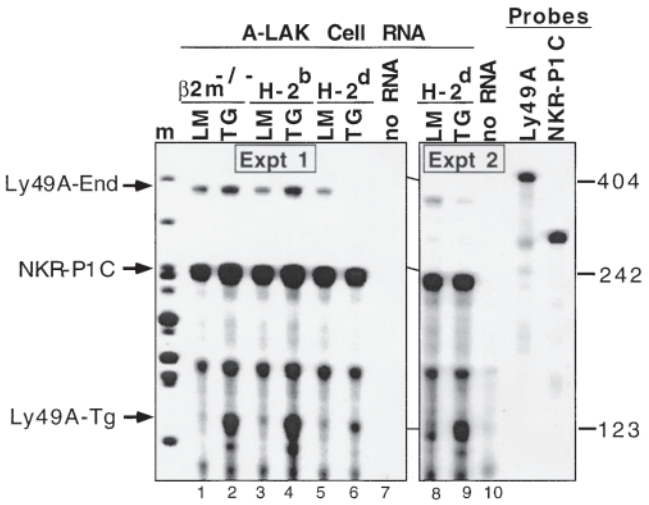

Figure 3. Ly49A transgene expression causes an MHC-dependent reduction in the levels of endogenous Ly49A mRNA. RNase protection assay of Ly49A mRNA levels and control NKRP1C levels in RNA preparations from NK cell (A-LAK) populations. One complete experiment and the H-2^d data from a second experiment are presented. The H-2^d transgenic sample in experiment 1 (lane *6*) was somewhat underloaded compared to the other samples, as shown by the lower intensity of the NKR-P1C band. When the phosphorimager values for the Ly49A endogenous band were normalized for NKR-P1C levels, comparable results to the other two experiments were obtained (see Table 3), and the Ly49A transgene band was comparable to the other transgenic samples. The protected RNA fragments are 379 nt for the endogenous Ly49A, 120 nt for the Ly49A transgene, and 239 nt for NKR-P1C. The 155-bp band present in all samples is from the NKR-P1C probe, and may represent transcript for another NKR-P1 isoform. The endogenous Ly49A mRNA levels in Ly49A transgenic H-2^d NK cells are significantly reduced compared to the levels in the nontransgenic littermate, based on a P <0.01 value using the two-tailed Student's t test (Table 3). *m*, markers.

cells coexpressing two or more self-specific Ly49 receptors. Thus, the hypothesis predicts that the Ly49A transgene, in H-2^d mice, should cause a reduced frequency of cells expressing endogenous, H-2^d–specific receptors, such as Ly49G2 and endogenous Ly49A.

In accord with this prediction, the percentage of NK cells expressing the H-2^d–specific Ly49G2 receptor (detected with 4D11 mAb) was reduced from 50.7% in nontransgenic H-2^d littermates to 18.5% in H-2^d line No. 2 Ly49A transgenic mice, a 2.7-fold reduction (Fig. 2, Table 2). A similar but slightly smaller effect was observed in the second transgenic line, line No. 12, where the frequency of Ly49G2$^+$ cells was reduced from 45% in nontransgenic H-2^{d+}

Table 3. *Relative Levels of Endogenous Ly49A mRNA Determined from RNase Protection Assays*

	Normalized mRNA levels					
	β2m$^{-/-}$		H-2^b		H-2^d	
NK cell sample	LM	Tg No. 2	LM	Tg No. 2	LM	Tg No. 2
Ly49A-end mRNA	100 ± 8	87 ± 16	92 ± 31	80 ± 10	76 ± 13	**30 ± 8***
Ly49A-end mRNA per Ly49A$^+$ cell	100 ± 8	NA	107 ± 36	NA	95 ± 16	NA

Average relative endogenous Ly49A mRNA levels ± SD from three independent experiments. cpm in relevant bands were determined with a phosphorimager. The cpm in the Ly49A band was divided by the cpm of the NKR-P1C control band in the same lane, and these values were normalized to the value obtained with the β2m$^{-/-}$ littermate preparation (assigned arbitrary value of 100 U). The calculated relative Ly49A mRNA levels per Ly49A$^+$ cell are also presented for the nontransgenic samples, determined by normalizing the relative mRNA levels to the average percentage of Ly49A$^+$ cells in each nontransgenic LAK cell sample, determined by flow cytometry (25.4% for β2m$^{-/-}$, 21.8% for H-2^b, and 20.4% for H-2^d samples). The substantial effect of the Ly49A transgene is indicated in bold. NA, not applicable.
‡P <0.01 versus H-2^d nontransgenic littermate by Student's two-tailed t test.

($H\text{-}2^{b/d}$) littermates to 22.3% in transgenic $H\text{-}2^{b/d}$ mice (Fig. 2, Table 2). It is of interest that this reduction in line No. 12 mice occurred, despite the significantly lower levels of transgene expression compared to line No. 2 mice. Both effects were highly reproducible and significant ($P < 0.001$). The similar effects observed in two transgenic lines represent strong evidence that the effects are due to MHC genes rather than to other genes the mice might have inherited from the transgenic founder mice despite multiple backcrosses. These results strongly support the hypothesis that an education process limits the number of cells coexpressing Ly49G2 and Ly49A in $H\text{-}2^{d}$ mice compared to $H\text{-}2^{b}$ or $\beta 2m^{-/-}$ mice. Much smaller, albeit statistically significant, reductions in the frequency of $SW5E6^{+}$ NK cells were also observed in $H\text{-}2^{d}$ mice of both transgenic lines (Table 2). Although these reductions are of dubious significance, they may occur because one of the two receptors reactive with the SW5E6 mAb, Ly49C, reportedly binds to $H\text{-}2^{d}$ class I molecules, whereas the other does not (16).

Surprisingly, in $H\text{-}2^{b}$ mice, which are normally thought not to express a Ly49A ligand (17, 25, 26), marginal, but statistically significant, reductions in the percentages of $Ly49G2^{+}$ cells were apparent in both Ly49A transgenic lines (Table 2). These reductions were only of 15–25%, and therefore of dubious significance. Because the percentage of $Ly49G2^{+}$ cells was not significantly affected by the transgene in $\beta 2m^{-/-}$ transgenic mice, the data suggest that the effect in $H\text{-}2^{b}$ mice may be due to an $H\text{-}2^{b}$–encoded class I molecule(s). Although Ly49A is not thought to react with $H\text{-}2^{b}$ class I molecules, the data raise the possibility that a weak interaction may occur. This interaction may be too weak to detect in functional experiments, yet, nevertheless, exert an effect in a developmental step, analogous to some T cell receptor–ligand interactions.

The hypothesized education process predicts that the Ly49A transgenes in $H\text{-}2^{d}$ mice should cause reduced usage, not only of Ly49G2, but also of endogenous Ly49A. Although we were unable to measure the frequency of cells expressing endogenous Ly49A in the transgenic mice by flow cytometry, we were able to determine the level of endogenous Ly49A mRNA with the use of the RNase protection assay. Considering first the results from nontransgenic mice, we observed that the average relative Ly49A mRNA level in $H\text{-}2^{d}$ mice (76 ± 13) was slightly lower than the level in $H\text{-}2^{b}$ mice (92 ± 31), which was slightly lower that the level in $\beta 2m^{-/-}$ mice (100 ± 8) (Table 3). These small differences could all be accounted for by a reduced frequency of $Ly49A^{+}$ cells observed in nontransgenic $H\text{-}2^{d}$ mice compared to non-transgenic $H\text{-}2^{b}$ and $\beta 2m^{-/-}$ mice (see Table 3). Thus, the level of Ly49A mRNA per $Ly49A^{+}$ cell was unaffected by MHC expression. Therefore, the reduced cell-surface expression of Ly49A observed in nontransgenic $H\text{-}2^{d}$ mice (13; Table 1) was not accompanied by reduced Ly49A mRNA levels per $Ly49A^{+}$ cell, supporting the contention that $H\text{-}2^{d}$–induced downregulation of Ly49A levels in normal mice is a posttranscriptional event. This observation is of interest in itself, and serves as an aid to interpretation of the transgenic data.

Analysis of the transgenic line No. 2 mice revealed that Ly49A transgene expression in $H\text{-}2^{d}$ mice resulted in a two- to threefold reduced level of endogenous Ly49A mRNA in the NK cell population compared to nontransgenic $H\text{-}2^{d}$ littermates (Fig. 3, Table 3). This reduction was reproducible and significant ($P < 0.01$). As previously noted, the transgene had little effect in $\beta 2m^{-/-}$ mice, nor did it have a significant effect in $H\text{-}2^{b}$ mice (Fig. 3, Table 3). Therefore, the reduction of endogenous Ly49A levels was dependent on both the transgene and $H\text{-}2^{d}$ expression.

The level of endogenous Ly49A mRNA in the population should correlate with the frequency of cells expressing endogenous Ly49A, as long as the amount of endogenous Ly49A mRNA per expressing cell remains roughly constant. As noted above, the level of Ly49A mRNA per $Ly49A^{+}$ cell in nontransgenic mice was unaffected by $H\text{-}2^{d}$ expression (Table 3). Since there is no apparent reason why the endogenous mRNA levels per cell should be downregulated by receptor–ligand engagement in transgenic mice, but not normal mice, we favor the interpretation that the reduced endogenous Ly49A levels in $H\text{-}2^{d}$ transgenic mice reflects a reduced frequency of cells expressing endogenous Ly49A. These data suggest that the transgene, in $H\text{-}2^{d}$ mice, results in a significant reduction in the proportion of cells expressing not only Ly49G2, but also endogenous Ly49A.

Discussion

Regulation of Ly49A Cell Surface Levels. The cell surface levels of Ly49 receptors are downregulated in mice that express the corresponding class I MHC ligands of the receptor (13, 25). It was proposed that receptor downregulation reflects a mechanism that calibrates Ly49 levels to the particular class I molecules expressed in the host (25). Our results demonstrate that Ly49A cell surface levels are regulated by a posttranscriptional event. First, despite the significant differences in Ly49A cell surface staining, the level of Ly49A mRNA per $Ly49A^{+}$ NK cell was the same in nontransgenic $H\text{-}2^{d}$ mice, where surface expression is low, as in nontransgenic $H\text{-}2^{b}$ or $\beta 2m^{-/-}$ mice, where surface expression is high (Fig. 3). Second, the $H\text{-}2^{d}$–induced downregulation of cell surface Ly49A expression was observed, even with a Ly49A transgene that was regulated by regulatory elements from a class I MHC gene and an immunoglobulin heavy chain gene (Fig. 1, Table 1). It should also be noted that the transgene does not contain the 3′ untranslated sequences found in the endogenous Ly49A transcript; 3′ untranslated sequences often serve as target sequences for regulation of transcript turnover rates (35). Considering that Ly49 receptor levels are downregulated in $H\text{-}2^{d}$ mice independently of the mRNA levels, the most likely explanation for the phenomenon would appear to be ligand-induced receptor modulation. We have not, however, ruled out the possibilities that receptor signaling at the cell surface causes inhibition of receptor protein biosynthesis from the mRNA, or prevents trafficking of the receptor to the cell surface. Previous evidence argues against the

possibility that downregulation is due to an intracellular interaction of Ly49A with D^d (36).

The receptor calibration model seems to imply that receptor levels are calibrated to some specific absolute level dependent upon the available class I molecules, to maximize sensitivity of the system to changes in class I levels. In light of this model, it is of interest that ligand-induced downregulation occurred even in the case of the Ly49A No. 12 transgenic line. In this line, transgene expression was generally lower than endogenous Ly49A expression, presumably due to the low transgene copy number and/or the integration site of the transgene. In H-2^b line No. 12 mice, the level of transgene-encoded Ly49A receptors was as low as the levels directed by the endogenous Ly49A gene or transgene No. 2 Ly49A receptors in H-2^d mice. In H-$2^{b/d}$ line No. 12 mice, the levels were reduced further. Since class I MHC levels did not change in the different transgenic lines, it cannot be said that the Ly49A receptor levels are set to a specific level dependent upon the interaction with MHC. These observations argue against the notion that receptor levels are calibrated to maximize sensitivity to changes in class I MHC levels (25). Rather, the results suggest that the presence of the ligand results in reduced levels of surface expression from the levels that pertain in the absence of the ligand. The altered Ly49A levels may nevertheless influence the functional class I specificity of the NK cell as has been previously proposed (25).

Transgenic Ly49 Gene Expression Does Not Prevent Endogenous Ly49 Gene Expression. Allelic exclusion of TCR-β and Ig heavy chain genes is accomplished by feedback inhibition, wherein expression of the protein product of one allele of the gene prevents activation of the other allele. For example, expression of TCR-β transgenes prevents activation of the corresponding endogenous genes, even when the transgenic mice do not express the MHC ligand of the transgenic receptor. In contrast, the Ly49A transgene had no significant effect on expression of the endogenous *Ly49A* genes in $\beta2m^{-/-}$ mice. Nor did the transgene affect the frequencies of cells expressing other Ly49 receptors in $\beta2m^{-/-}$ mice. The results argue strongly against a model in which *Ly49A* gene expression is inhibited by the mere presence of Ly49A protein in the cell, independent of the presence of a corresponding class I ligand. Therefore, monoallelic *Ly49A* gene expression must have another explanation. In light of this, the issue arises of whether monoallelic *Ly49A* gene expression should be called "allelic exclusion," as we have previously called it. It should be noted that an "unregulated" model was once prominently proposed to account for allelic exclusion of immunoglobulin genes, suggesting that the term does not imply a specific mechanism (37). Furthermore, it remains possible that regulation of the extent of monoallelic gene expression occurs in mice that express the corresponding MHC ligand (see below). In any case, this issue is primarily a semantic one.

As we have proposed elsewhere, one possibility to account for monoallelic *Ly49* gene expression is that during NK cell development, *Ly49* genes are chosen for expression by a stochastic process (12, 19). If different *Ly49* genes, and different alleles of the same *Ly49* gene, are activated independently and with a relatively low probability, most cells would activate only one of the two alleles at a given *Ly49* locus. This model would also account for the fact that NK cells commonly coexpress two or more different Ly49 receptors. This mechanism would ensure that different cells express different combinations of Ly49 receptors, and thus would serve to generate clonal diversity. A repertoire generated in this way might then be modified by an MHC-dependent education process.

MHC-dependent Education Process. The Ly49A transgenic mice provided the opportunity to test the hypothesis that an education process limits the number of NK cells that coexpress multiple self-specific Ly49 receptors. This hypothesis was based on analysis of the repertoire in nontransgenic mice, which revealed that coexpression of two H-2^d–specific Ly49 receptors, Ly49A and Ly49G2, occurred less frequently in H-2^d mice than in mice that do not express H-2^d (12). The finding that Ly49A transgene expression in all NK cells caused a specific reduction in the frequency of $Ly49G2^+$ NK cells in H-2^d mice, and to a much smaller extent in H-2^b mice, but not in $\beta2m^{-/-}$ mice (Fig. 2) fulfills a major prediction of this model. The observation that the low expressing (line No. 12) and high expressing (line No. 2) transgenic lines exhibited a comparable reduction in $Ly49G2^+$ cells in H-2^{d+} mice suggests that the Ly49A–D^d interaction is sufficiently strong that even low Ly49A surface expression can cause these alterations in the repertoire. The comparable results with two transgenic lines also demonstrates that the results were a consequence of the transgene, rather than of other genes coincidentally inherited from the transgenic founders.

The hypothesis that an education process limits coexpression of multiple self-specific receptors also predicts that endogenous *Ly49A* gene usage should be less frequent in the Ly49A transgenic mice. The finding that the H-2^d transgenic NK cells contained less endogenous Ly49A mRNA than nontransgenic mice or than transgenic H-2^b and $\beta2m^{-/-}$ mice (Table 3) is consistent with the hypothesis. The reduction in endogenous Ly49A mRNA among NK cells in H-2^d transgenic mice could reflect a reduction in the amount of mRNA per expressing cell, rather than a reduction in the frequency of expressing cells. However, the finding that H-2^d expression does not affect the level of Ly49A mRNA per $Ly49A^+$ cell in nontransgenic mice (Fig. 3) argues against a ligand-dependent mechanism that reduces the mRNA levels in each expressing cell. Therefore, although not definitive, the results are most consistent with the interpretation that NK cells expressing endogenous Ly49A are less frequent in transgenic H-2^d mice.

The effects of transgene expression on endogenous Ly49G2 and Ly49A gene usage strongly suggest that an education process disfavors NK cells that express multiple self class I–specific Ly49 genes or alleles. Why should the education process disfavor NK cells that coexpress multiple self–class I–specific Ly49 receptors? Previous analysis of NK cells that coexpress two Ly49 receptors suggests that each Ly49 receptor can independently inhibit NK cell lysis (10, 38). If each

NK cell expressed all self–class I–specific receptors, the NK cell population would be unable to attack potential target cells that have extinguished expression of some, but not all, self–class I molecules. We propose that by imposing a limit on the coexpression of self–class I–specific receptors, the education process greatly enhances the discriminatory capacity of the NK cell population for self–class I molecules.

We have previously proposed two models of NK cell education that address how MHC class I expression may shape the Ly49 receptor repertoire (12, 19). In a selection model, the stochastic activation of receptor alleles in different cells during an initial stage of NK cell development, generates clonal diversity. This step is followed by MHC-dependent selection steps that favor the most "useful" NK cells. It was proposed that one such selection step would ensure that each NK cell expresses at least one self–class I–specific inhibitory receptor, thus preventing autoimmunity. To account for the findings reported here and previously (12), it must also be argued that the selection process includes a step in which cells expressing multiple self–class I–specific receptors are selected against. This process could account for the effects of the transgene observed in this study (39).

Alternatively, a sequential receptor activation model suggests that *Ly49* receptor genes may undergo a stage of stochastic activation where different receptors are successively and cumulatively displayed on the cell surface; when the cell eventually expresses a sufficient number of self–class I–specific receptors to achieve a defined threshold of Ly49 receptor signal, the *Ly49* gene activation mechanism is disabled, fixing the Ly49 receptor pattern of the cell (12). This model predicts that the formation of NK cells expressing excess self–class I–specific receptors will not occur. Such a mechanism would result in a decreased frequency of NK cells expressing specific pairs of self-specific Ly49 receptors, consistent with our data (39).

Can these models be distinguished? One possible difference in the predictions of the two models concerns the effects of the transgene on non-self–specific receptors (39). The selection model predicts that the transgene should only affect usage of other self-specific receptors, and not the usage of non-self–specific receptors. In contrast, the sequential receptor activation model would seem to predict that early expression of Ly49A on all NK cells in H-2^d mice will result in decreased usage of any other receptor, whether it be self or non-self specific. This is because early expression of Ly49A should result in an earlier termination of new receptor expression. The data presented herein demonstrates that in H-2^d mice, the transgene exerted a larger effect on Ly49G2 usage than on Ly49C/I receptor usage detected with the SW-5E6 mAb (Table 2). Although Ly49C is thought to bind to H-2^d class I molecules, Ly49I reportedly does not (40). Thus, these data might seem to refute the sequential model. However, the predictions of the sequential model would need to be modified if there turns out to be a defined order in which different Ly49 receptors are expressed during development. Moreover, the precise functional specificities of Ly49C and Ly49I are still under investigation. Therefore, we believe that it is premature to arrive at a conclusion on this point based on the available data.

The results reported here clearly demonstrate that a class I–dependent NK cell education process occurs. However, they do not yet discriminate between the selection and sequential receptor activation models, and other models that might be envisaged to explain NK cell education. The production of new reagents that detect each of the Ly49 receptors, and a thorough knowledge of the specificity and expression patterns of each of the receptors, should facilitate this endeavor. Furthermore, elucidation of the molecular mechanisms that activate *Ly49* genes in different NK cells, of considerable interest in itself, may also suggest approaches to generate mice that express a more limited Ly49 repertoire. Such mice would represent useful tools for analysis of NK cell development.

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